PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/18, C07K 14/48, 14/475 — A1

(11) International Publication Number: **WO 97/14427**

(43) International Publication Date: 24 April 1997 (24.04.97)

(21) International Application Number: PCT/SE96/01305

(22) International Filing Date: 15 October 1996 (15.10.96)

(30) Priority Data:
9503620-8 17 October 1995 (17.10.95) SE

(71)(72) Applicant and Inventor: HAGLID, Kenneth, G. [SE/SE]; Gärdesvägen 18, S-436 51 Hovås (SE).

(74) Agents: GRAUDUMS, Valdis et al.; Albihn West AB, P.O. Box 142, S-401 22 Göteborg (SE).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
In English translation (filed in Swedish).

(54) Title: USE OF PROTEIN S-100b IN MEDICINES AND MEDICINES CONTAINING THE PROTEIN S-100b

(57) Abstract

The present invention concerns the use of the protein S-100b in medicines for the stimulation of growth and survival of damaged neurons. The invention includes as well a medicine containing the S-100b protein in an aqueous solution which may contain also other biocompatible substances.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

TITLE:
Use of protein S-100b in medicines and medicines containing the protein S100-b

AREA OF THE INVENTION:
The present invention concerns the use of a protein S100-b in medicines which aim at stimulating the growth of as well as survival of damaged neurons by primarily the administration of a medicine containing this protein via infusion or injection locally to the damaged region.

BACKGROUND OF THE INVENTION:
Structure of normal peripheral nerves, kranial nerves, spinal cord and rhombencephalon.
Peripheral nerves and cranial nerves as well as the spinal cord constitute a functional unit which is responsible for the movements of the body. The unit includes also sensory impulses to the cerebrum and the cerebellum. When undamaged, the unit provides information directed inwards and outwards which is a prerequisite for our daily life.

Each neuron consists of a cell body (perikaryon or soma) as well as two types of processes, one which receives information (dendrites) and one which delivers information (axon) as electrical impulses. The axons of peripheral nerves and cranial nerves are contained within Schwann cells which produce myelin, an insulating lipid-rich material which isolates each axon from the others. In the spinal cord and the rhombencephalon, oligodendroglial cells surround the axons and form the myelin sheaths. In addition, other cell types, such as astrocytes and microglial cells, are present in the spinal cord.

Astrocytes make up 50-70% of the volume occupied by glial cells in the brain. They maintain the microenvironment of the neurons. The microglial cells are much fewer. They are activated during damage to the brain and phagocytize dead material. Furthermore, they play a key role in the immune system of the brain.

Organization of the peripheral nerves and the spinal cord.

The peripheral nerves are made up of axons which mediate impulses to the muscles which in turn cause contractions (motor axons) and axons which mediate information from muscle spindles, tendons joint capsules and the skin (sensory axons). The motor axons are the processes from large perikarya localized in the anterior horns of the spinal cord. The sensory axons have their perikarya in the dorsal root ganglia where they are surrounded by satellite glial cells. The sensory neurons in the spinal cord transfer the information further to the cerebellum and cerebrum.

Structure of the spinal cord.

The neuronal perikarya of the spinal cord are organized as grey matter which, in the cross section, is shaped like an H. This is surrounded by white matter which contains axons travelling up or down.

Structure of rhombencephalon and the cranial nerves.

Ten out of the twelve cranial nerves have their perikarya located in groups (nuclei) within the rhombencephalon. The cranial nerves have either a motor or a sensory function.

REACTION TO DAMAGE:

Inflammation and cellular response to damage.

Damage to brain tissue leads to an inflammatory response which is characterized by a vascular response as well as a cellular response, both aiming at defending the body

against foreign substances and to dispose of dead and dying tissue. The inflammation per se also prepares the tissue for the process of repair. If axons are sewered, their peripheral parts consistently degenerate. The proximal portions of certain axons, as well as their perikarya degenerate as well and die.

The magnitude of the inflammatory and degenerative processes depends on the size of the damage, the condition of the tissue, as well as on the capacity of the body to provide a response. The primary sign of initiation of the repair process is the growth of axons from surviving perikarya and the elongation of surviving proximal portions of axons. These axons grow through the damaged region and into the sheaths of the degenerated distal part of the nerve or into the spinal cord. This process is slow (a few millimeters per day) and the result of it is uncertain, since the target organs (muscles, blood vessels, skin, tendons, joints, cartilage, bone) which have not received nerve pulses for some time, tend to degenerate prior to the arrival of the growing axons. This leads to a reduced muscle power or paralysis as well as reduced sensory information which indeed constitutes a severe handicap for the affected individual. This type of damage is a considerable socio-economic drawback also for the society.

Cellular phases of nerve tissue repair.

Two principal types of damage may appear. The first is due to compression of the nerve tissue (axon), while its myelin sheath (basal membranes, endo, peri and epineurium) which surrounds the dying axon remains intact. The second type is due to a loss of nerve tissue, in which case there is a gap between the proximal part and peripheral part of the axon. Repair of this type of nerve damage requires bridging constructs (silicon chambers, transplants of nerves or other tissues). The cellular response is similar in the two

types of damage. The degenerating tissue is first invaded by macrophages which initially remove damaged components and dying material. The damaged region is invaded by fibroblasts and small blood vessels within a few days. The Schwann cells then grow and invade the damaged region from the proximal end of the nerve. Then axons from the surviving perikarya grow in close association with the Schwann cells.

A considerable amont of knowledge has accumulated during the last few decades concerning the mechanisms which regulate the activity of the cells during the process of repair. Chemotactic peptides have been isolated and the role of prostaglandins and related compounds is partly clarified. The details of the steps in the processes which lead to improved repair and regeneration are, however, largely unknown.

EVALUATION OF PRESENTLY EMPLOYED METHODS FOR THE REPAIR OF DAMAGE AND THEIR LIMITATIONS:

Peripheral nerves

A number of different techniques are employed for the repair of damage on peripheral nerves and cranial nerves. In the case of a clearcut sewering, the nerve stumps are connected by suturing. There are more problems when part of the nerve is lost. The presence of granulation tissue and collagen usually prevent a functional tissue repair process. This is largely due to a deficit of axons which can bridge over to the peripheral part of the nerve, i.e. that which leads to the target organs.

When part of the nerve is missing, a transplant has to be sutured to bridge the proximal with the peripheral nerve stumps. A silicon tubing or a nerve transplant is mostly employed. Presently tests are performed with muscle transplants which have been made acellular by repeated

freezing and thawing (liqiud nitrogen - room temperature). However, the results are still fairly poor.

The degree of growth of axons and Schwann cells may be stimulated by the administration of growth factors.

CONCLUSION:
In the case of a large damage to peripheral nerves, cranial nerves or the spinal cord, the regeneration capacity is poor or absent and the result of the repair process is a reduced functional capacity. Small nerve damages, on the other hand, cause usually negligible defects. Consequently, there is a considerable need for improvement of the repair process after large damages. Such improvements may also be helpful for the repair of minor damage.

A number of factors which promote or reduce the rate of tissue repair have been described during the last decade. Many of these derive from tumors or are associated with tumors (oncogens). Some of these products stimulate growth, either in an unspecific fashion or directed to a specific type of tissue
or organ. A considerable problem is that some of these compounds may induce transformations and/or have unwished side-effects.

<u>Criteria for a neurotrophic factor.</u>
A neurotrophic factor stimulates growth of nervous tissue. The following criteria has to be fulfilled by a substance in order to classify it as a neurotrophic factor (NTF).

1. A neuron which employs NTF should contain NTF and have receptors for NTF.
2. The neuron should survive in the presence of NTF and die in its absence.
3. A machinery for the synthesis of NTF should be present

in the target cells or in other cells in contact with the neuron. 4. NTF should support the elongation of those processes which take place in those specific groups of cells which fulfil criterium 1.

S-100b as a neurotrophic factor

The S-100 family, i.e. S-100a, S100b, S-100L, S-100G, calpactin LC, calcylin and calbindin9K are small, acid, calcium-binding proteins which probably are involved in the progression of the cell cycle, in cell differentiation and in the interactions between the cytoskeleton and the plasma membrane of the cell. The "original" S-100 molecule was discovered 30 years ago and was given its name due to its solubility in 100% ammonium sulphate. S-100 has three dimeric isoforms, S-100a0, S-100a and S-100ab, which are formed by $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ subunits. S-100b dominates quantitatively in mammalian brains and is the only component in the rat brain.

The presence or not of S-100 in neurons is a controversial question since 20 years. One reason is that tissue fixation with aldehydes tends to mask the antigenic properties of S-100b. In my research, I have recently ascertained the presence of S-100b in certain populations of neurons in the rhombencephalon of the rat. In this discovery, I have employed a new method for the expression of the S-100b antigen (Yang, Q Hamberger, A. Hyden, H. Wang, S. Stigbrand, T and Haglid, K.G., S-100b has a neuronal localization in the rat hindbrain revealed by an antigen retrieval method, Brain Res., 696 (1995) 49 61.)

The S-100 immunoreactivity of neurons in the developing brains of, in particular rodents, was generally overlooked in previous work, largely due to the choice in those studies of brain regions and/or of stages of development of the animal.

Only part of the neuronal population contains S-100b or has receptors for S 100b. I have described above a method which has the capacity to determine which neurons contain the protein or its receptors. I have shown that, in the rat, the presence of S-100b increases postnatally in certain neurons and that a large number of these neurons contains S-100b or has receptors for S-100b on their surface. In the adult rat, the S-100b containing neurons are found in the mesencephalon, namely in the red nucleus, the oculomotor nucleus, the mesencephalic trigeminal nucleus, in the pons, namely the pontine reticular nucleus: -oral, -caudal, -ventral, and the motor trigeminal nucleus, in the medulla oblongata, namely in the facial nucleus, the vestibular and lateral vestibular nuclei, in the cerebellum, namely in the cerebellar nuclei, in the spinal cord, namely in the motor neurons and, finally, in the sensory neurons of the dorsal root ganglia. These groups of neurons represent examples of neurons which may be influenced by the medicine according to the present discovery. In addition, other neurons may be influenced by the medicine.

THE PROBLEM:

Neurological expertise has estimated that a large number of damages to peripheral nerves, cranial nerves and the spinal cord heal incompletely, i.e. the target organs degenerate or undergo fibrotic changes. Such complications give frequently functional handicaps as well as social disturbancies and reduced working capacity. One of the most important goals of biomedical research is, since many years, an improved and more rapid healing of damage to the nervous system.

THE SOLUTION:

According to the present invention, the mentioned problem is considerably reduced, since the use of the S-100b protein in medicines stimulates the growth of nerves and

promotes the survival of damaged neurons.

According to the invention, the S-100b protein is useful for neurons which have receptors for S-100b and/or contain S-100b.

The invention concerns also the use of the S-100b protein for neurons which have been damaged by diseases such as amytrofic lateral sclerosis and multiple sclerosis.

The invention concerns also a medicine which contains the S-100b protein for stimulation of nerve growth and the survival of damaged neurons.

According to the invention, the medicine may contain S-100b in a concentration of 0.1 to 1000 μg/ml in a water solution which may contain also other biocompatible substances.

According to the invention, the medicine shall be administered by infusion or injection with e.g. a mini-osmotic pump to the damaged region.

There are no indications that S-100b has oncogenic or oncogen-related properties. S-100b is an endogenous peptide which may be produced with known biotechnological methods. S-100b has a strictly local effect and has hardly any side effects after local administration.

S-100b is, as mentioned above, a peptide with the following amino acid sequence of the monomer subunit (β):
Met Ser Glu Leu Glu Lys Ala Met Val Ala Leu. Ile Asp Val Phe His Gln Tyr Ser Gly Arg Glu Gly Asp Lys His Lys Leu Lys Lys Ser Glu Leu Lys Glu Leu Ile Asn Asn Glu Leu Ser His Phe Leu Glu Glu I le Lys Glu Gln Glu Val Val Asp Lys Val Met

Glu Thr Leu Asp Glu Asp Gly AspGly Glu Cys Asp Phe Gln Glu Phe Met Ala Phe Val Ser Met Val Thr Thr Ala Cys His Glu Phe Phe Glu His Glu

Consequently, S-100b reduces the time required for nerve regeneration. This reduces, in turn, the functional and social incapacity of the patient, since the rehabilitation period after a major nerve damage is shortened. This reduces problems arising from muscle inactivity and changes in the structure of bones and other organs. The dose of S-100 which is required, varies within a wide range and is determined by the type, degree and localization of the damaged tissue. Treatment is required for days or weeks.

Examples of the use of S-100b and medicines containing S-100b for local administration to rats in which the sciatic nerve previously had been damaged and a piece of it removed, are given below.

Examples

1.S-100b was used at a concentration of 0.5-1000 µg/ml in a salt solution which was buffered with 100 mM phosphate, had a pH of 7.2 and contained 2.5 mM $CaCl_2$ as well.

2.S-100b was used at a concentration of 0.1-1000 µg/ml in 1% bovine serum albumin (BSA) in a buffered salt solution which also contained 2.5 mM $CaCl_2$.

3.S-100b was used at a concentration of 0.5-1000 µg/ml in a salt solution which also contained 2.5 mM $CaCl_2$.

4.S-100b was administered in a gel made of hydroxy-methyl-cellulose at a concentration of 25 µg/ml (made by Apoteksbolaget AB, Umeå, Sweden).

5.S-100b was dissolved in a gel made of

hydroxy-methyl-cellulose at a concentration of 10 µg/ml.

6.S-100b was dissolved to a concentration of 2.5 µg/ml in a gel made of 3% hydroxy-methyl-cellulose.

7.S-100b was administered with an osmotic minipump in a concentration of 0.5-10 µg/ml to the region of a damaged sciatic nerve in rats. A 10 mm portion of the sciatic nerve was removed and replaced by an acellular (frozen-thawed) muscle transplant which bridged the proximal and the distal nerve stumps. Twelve rats were used as controls and to these the solution without S 100b was administered. Eleven rats were given S-100b (0,57 µg/h) for 6 days with an Alzets osmotic minipump. The length of nerve regeneration into the acellular transplant was determied on the sixth day with a pinch test. The results are shown below.

Mann-Whitney U for pinch
Grouping Variable: grupp

U	11.000
U Prime	121.000
Z-Value	-3.385
P-Value	.0007
Tied Z-Value	-3.391
Tied P-Value	.0007
# Ties	4

Mann-Whitney Rank Info for pinch
Grouping Variable: grupp

	Count	Sum Ranks	Mean Rank
ha	11	187.000	17.000
kontroll	12	89.000	7.417

The "MeanRank" value of 17.000 was calculated for the rats which had been treated with S-100b (lower right of the table) while the corresponding controls had the value of 7.417. The ratio of these two numbers shows that the axons

which had been treated with the medicine according to the present discovery had grown 2.3 times more than the unstimulated control axons. The probability test of the significance of this difference gave a P-value of less than 0.0007.

Consequently, medicines which contain S-100b improves the regeneration and repair as well as survival of different types of nerve tissue. Accordingly, not only the growth of the axons is stimulated but the treatment also increases the capacity for survival of the neurons.

The invention is not limited to the examples given above but can be varied in different ways within the frame of the patent claims.

CLAIMS

1. The use of the S-100b protein in medicines which stimulate the growth and survival of damaged neurons.

2. The use according to claim 1 for the treatment of neurons which have receptors for S-100b and/or contain S-100b.

3. The use of S-100b, according to any of the claims 1-2 for neurons which have been damaged by diseases such as amyotrophic lateral sclerosis or multiple sclerosis.

4. Medicines containing the S-100b protein for the stimulation of growth and survival of damaged neurons.

5. Medicines according to claim 4, in which S-100b is used in a concentration of 0.1-100 µg/ ml in a water solution which may contain also other biocompatible substances.

6. Administration of the medicine according to any of the claims 4 or 5 by infusion or injection to the damaged region with the use of e.g. an osmotic minipump.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 96/01305

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 38/18, C07K 14/48, C07K 14/475
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI, WPIL, US PATENTS FULLTEXT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9210200 A1 (NEW YORK UNIVERSITY), 25 June 1992 (25.06.92), page 19, line 2 - line 8, claim 3 -- --------	1-6

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" erlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 January 1997	01 -02- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 96/01305

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 6
because they relate to subject matter not required to be searched by this Authority, namely:
See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by therapy.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

28/10/96

International application No.
PCT/SE 96/01305

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9210200	25/06/92	AU-A- 9164591	08/07/92

Form PCT/ISA/210 (patent family annex) (July 1992)